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Award Number: W81XWH-10-1-0917

TITLE: VRP09 Reduction of Corneal Scarring Following Blast and Burn Injuries to Cornea Using siRNAs Targeting TGFb and CTGF

PRINCIPAL INVESTIGATOR: Gregory Schultz, Ph.D.

CONTRACTING ORGANIZATION: University of Florida
Gainesville, Florida 32611-5500

REPORT DATE: October 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2011	Annual	27 September 2010 – 26 September 2011

4. TITLE AND SUBTITLE

VRP09 Reduction of Corneal Scarring Following Blast and Burn Injuries to Cornea Using siRNAs Targeting TGFb and CTGF

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0917

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Gregory Schultz

E-Mail: schultzg@ufl.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Florida
Gainesville, Florida 32611-5500

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Blast and burn injuries to the eye caused by explosions during combat or terrorist attacks are devastating injuries, which typically impair vision by excessive corneal scarring. Our overall goal is to develop a topical therapy that will reduce corneal scarring by selectively reducing expression of TGFb, TGFb receptor-II (TGFBRII), and CTGF genes which cause scarring using the newly discovered effects of small interfering RNAs (siRNA). In the first year of this project, we designed and tested at least three siRNAs targeting each of the three target gene mRNAs using cultures of rabbit corneal fibroblasts (RCF). The optimal siRNA for each gene was then formulated into double and triple combinations, and one triple combination of three siRNAs knocked down expression of collagen gene by 97% in RCF cultures without compromising the viability of the RCF. This triple combination of siRNAs will be the lead formulation that we will test in vivo using the rabbit corneal excimer laser ablation model that simulates blast injuries during the next year.

15. SUBJECT TERMS
None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	21	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Blast and burn injuries to the eye caused by explosions during combat operations or terrorist attacks are devastating injuries, and in eyes that can be saved, the major causes of vision impairment are excessive corneal scarring and neovascularization. Unfortunately, no approved drugs have been shown to improve vision outcome in eyes with these types of corneal injuries. However, decades of clinical experience and laboratory research have shown that the key to improving vision outcome is to improve the quality of corneal wound healing.[1], [2], [3] Our overall objective is to develop new drugs that use the emerging technology of RNA-interference (RNAi) to reduce vision impairment following corneal injuries by reducing expression of genes that stimulate formation of corneal scar (corneal haze).[4] We reported previously that corneal scarring is primarily up-regulated by the actions of transforming growth factor beta (TGFb), which stimulates corneal cells by binding to the TGFb type II receptor (TGFbRII) and inducing expression of connective tissue growth factor (CTGF).[5] CTGF then directly up-regulates synthesis of collagen scar and induces transformation of fibroblasts into myofibroblasts. Our approach is to design and test small interfering RNAs (siRNA) that will selectively reduce the level of expression of these three key proteins that stimulate corneal scar formation, and thereby, reduce vision loss. We focused on siRNAs because they are the most potent and selective of all gene-targeted, oligonucleotide-based drug approaches (better than ribozymes, antisense oligonucleotides (ASO), or microRNAs).[4] We will accomplish this objective in three specific aims. First, we will design and test siRNAs that selectively target the mRNAs of each of the three target genes, TGFb, TGFbRII and CTGF, using cultures of rabbit corneal fibroblasts (RCF). We will then test the optimal siRNA for each gene when formulated into double and triple combinations using cultures of RCF to obtain the maximum knock-down of collagen synthesis. Second, we will test the two most effective siRNA combinations for reduction of corneal scarring (haze) using the rabbit models of blast and burn corneal injuries. Third, we will compare the effectiveness of siRNAs oligonucleotides and AAV-vectored siRNAs in rabbit models of blast and burn corneal injuries for reduction of corneal scarring (haze).

BODY

We accomplished all the primary objectives for the first year of this project, and we are on schedule to begin the next phase of developing an effective antiscarring drug therapy for corneal blast and burn injuries. We established primary cultures of RCF that we then used to test the siRNAs. We designed and tested at least three siRNAs at that target each of the three target gene mRNAs (TGFb, TGFbRII and CTGF) at multiple concentrations in the RCF cultures. We identified at least one siRNA that knocked-down the level of mRNA at least 70% for each of the target genes in the cultures of RCF. These results were confirmed by measuring the levels of the target proteins in the medium and in extracts of the RCF. We then combined the optimal siRNA for each of the target genes in pairs of two siRNAs and tested the effect on knock-down of target mRNAs. Finally, we formulated a triple combination of siRNAs targeting each the three target genes based on the results from tests of the single and double combination of siRNAs, and tested its ability to knock-down levels of each of the three target genes mRNAs and proteins, and most importantly, to knock-down the level of type-I collagen mRNA in cultures of RCF. We achieved a tremendously positive result, with the triple combination of siRNAs reducing the level of type-I collagen mRNA by 97%, without any general toxic effect on the RCF. This is a very significant accomplishment that will allow us to proceed with the next phase of the research, which is to begin testing the triple combination of siRNAs in the rabbit corneal excimer laser ablation model that simulates blast injuries during the next year.

Objective 1a. Establish primary cultures of rabbit corneal fibroblasts for use in assessing effects of siRNAs on expression of the three target genes.

We successfully established cultures of rabbit corneal fibroblasts using a procedure we described previously in which fresh rabbit corneas were obtained and rinsed extensively to remove bacteria and fungi then chopped into small pieces ~1 mm^3 and placed into T-25 culture flasks with medium containing 20% fetal calf serum. After ~1 week the fibroblasts migrate from the corneal blocks and proliferate. When initial confluency is reached the cultures are split with trypsin/EDTA and seeded into 48 well or 96 well culture plates for use in testing the siRNAs.

Objective 1b. Optimize conditions for transfection of preformed siRNAs into cultures of rabbit corneal fibroblasts.

As shown in Figure 1, several different conditions were evaluated for optimal transfection of preformed siRNAs into low passage cultures of RCF. Initial transfection optimization of siRNAs into rabbit cornea fibroblast (RCF) was performed using the Ambion *Silencer*® siRNA Starter Kit. The kit supplies a GAPDH siRNA and several methods to analyze the concentration of GAPDH after transfection of the GAPDH siRNA. Two different siRNA transfection reagents, siPORT™ *NeoFX*™ Transfection Agent and *Trans*IT-TKO® Transfection Reagent, were used in different quantities to determine which transfection conditions were best suited for RCF transfection. The siPORT™ *NeoFX*™ Transfection Agent produced the greatest knockdown of GAPDH at approximately 17% when using 0.5 ul of the siPORT™ *NeoFX*™ Transfection Agent. Based on these results, we routinely used 0.5 ul of siPORT™ *NeoFX*™ Transfection Agent to transfect the RCF. Low levels of CTGF, TGF-B1 and TGF-B2R were found in the RCF. Our laboratory has also optimized the stimulation of these cells to produce a greater of amount of these growth factors by adding low levels of estradiol to increase TGFb and TGFbRII mRNAs, and adding low levels of TGFb1 to up-regulate CTGF mRNA. This stimulation simulates the up regulation of CTGF, TGF-B1 and TGFbRII in the cornea after an injury.

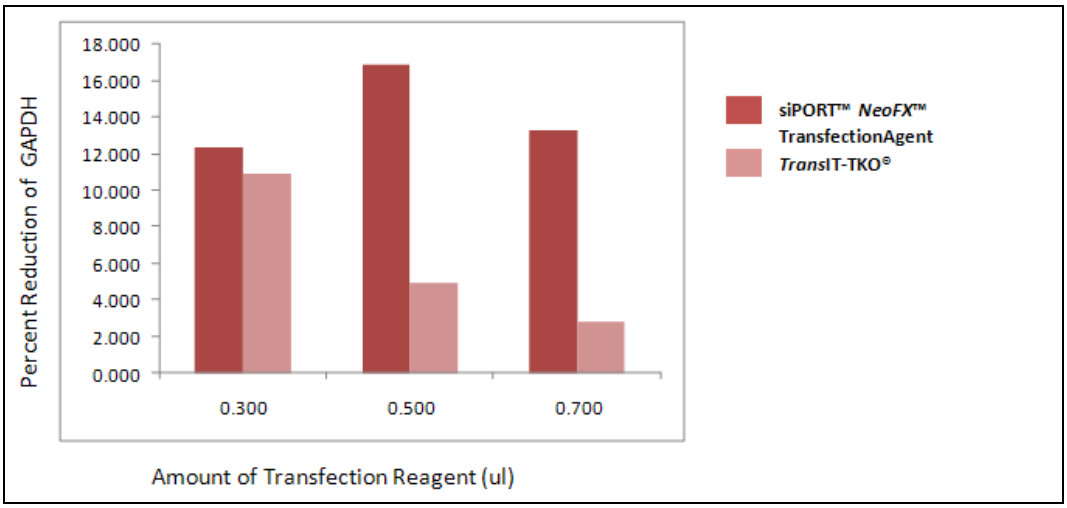

Figure 1. *Optimization of conditions to transfect cultures of Rabbit Corneal Fibroblasts with siRNAs.*

Objective 1c. Design up to four siRNA oligonucleotides (22mers) that selectively target mRNAs for each of three key genes that regulate corneal scarring: TGFb, TGFbRII, and CTGF.

As shown in Table 1, below, we designed siRNAs for all tree target mRNAs using algorithms that were provided by commercial companies and that are available through free software programs. These siRNAs have all been ordered from the manufactures indicated and will be delivered in the next few weeks.

Table 1		**siRNA sequences**		
Target TGFb1	**Sense/Antisense**	**Production_Sequence**	**Start**	
	s	CCAACAUGAUCGUGCGCUCdTdT	305	Sigma & Dharmacon
	a	GAGCGCACGAUCAUGUUGGdTdT	305	
	s	GAGCAGCUGUCCAACAUGAdTdT	295	Sigma & Dharmacon
	a	UCAUGUUGGACAGCUGCUCdTdT	295	
	s	GCAGCUGUCCAACAUGAUCdTdT	297	Sigma & Dharmacon
	a	GAUCAUGUUGGACAGCUGCdTdT	297	
	s	CUACUGCUUCAGCUCCACAUU	13	Ambion/Dharmacon
	a	UGUGGAGCUGAAGCAGUAGUU	13	
Target TGFbRII	**Sense/Antisense**	**Production_Sequence**	**Start**	
	s	GGAAAGAACATGTGAGCAA	7177	Ambion - 7171
	a			
	s	CGACAGGACTATAAAGATA	7313	Ambion - 7318
	a			
	s	CAAACTACCTACAGAGATT	3589	Ambion - 3590
	a			
	s	CAACTAGAATGCAGTGAAA	5033	Ambion - 5034
Target CTGF	**Sense/Antisense**	**Production_Sequence**	**Start**	
	s	AAGCTGACCTGGAAGAGAA	752	Ambion/Dharmacon
	a			
	s	AAGAAGAGCATGATGTTCA	964	AmbionDharmacon
	a			
	s	AAGAAGGGCAAGAAGTGCA	775	Ambion/Dharmacon
	a			
	s	TGGAAGAGAACATTAAGAA	761	Ambion - 764
	a			

Objective 1d. Design and test Q-RT-PCR primers for each of three key genes that regulate corneal scarring: TGFb, TGFbRII, and CTGF and the control house keeping gene GAPDH.

As shown in Table 2, we also designed and ordered primers for the three genes that will be used in Q-RT-PCR assays to measure the levels of the mRNAs in cultures of cells treated with the siRNAs as described below and in rabbit corneas following excimer ablation (blast injury) and thermal injury (burn injury).

TABLE 2		RT-PCR Primers						
CTGF								
Name	Sequence	Start Posit	Strand	Length	Primer Tm	Purity	Modification	Scale
Primer Set 1: Amplicon Size = 82								
AB217855.1_L1	AGGAGTGGGTGTGTGATGAG	300	forward	20	58.51	GAP		0.05 umol
AB217855.1_R1	CCAAATGTGTCTTCCAGTCG	362	reverse	20	59.13	GAP		0.05 umol
AB217855.1_P1	ACCACACCGTGGTTGGCCCT	327	forward	20	69.25	HPLC	5'Fam - 3'Tamra	0.05 umol
TGF B1								
Primer Set 2: Amplicon Size = 86								
AF000133.1_L1	CCTGTACAACCAGCACAACC	189	forward	20	59.06	GAP		0.05 umol
AF000133.1_R1	CGTAGTACACGATGGGCAGT	255	reverse	20	58.68	GAP		0.05 umol
AF000133.1_P1	CTCCAGCGCCTGTGGCACAC	233	reverse	20	69.92	HPLC	5'Fam - 3'Tamra	0.05 umol
TGFBR2								
Primer Set 1: Amplicon Size = 109								
BD061291.1_L1	CGTCGAGACTCCATCTCAAA	4665	forward	20	58.96	GAP		0.05 umol
BD061291.1_R1	AAACAGCCCACAAATGTCAA	4754	reverse	20	59.02	GAP		0.05 umol
BD061291.1_P1	TCAGCTTTGCACAAGGGCCCT	4713	reverse	21	68.48	HPLC	5'Fam - 3'Tamra	0.05 umol
GAPDH								
Primer Set 2: Amplicon Size = 94								
NM_001082253.1_L1	GAGACACGATGGTGAAGGTC	67	forward	20	58.05	GAP		0.05 umol
NM_001082253.1_R1	ACAACATCCACTTTGCCAGA	141	reverse	20	59.14	GAP		0.05 umol
NM_001082253.1_P1	CCAATGCGGCCAAATCCGTT	93	reverse	20	69.17	HPLC	5'Fam - 3'Tamra	0.05 umol

Objective 1e. Test up to four siRNA oligonucleotides (22mers) that selectively target mRNA for TGFb1 in low passage cultures of rabbit corneal fibroblasts for knockdown of TGFb1 protein and mRNA.

As shown in Figure 2, below, three different siRNAs targeting TGFb1 mRNA were tested for knockdown of TGFb1 protein using the optimized transfection conditions that were established in experiments performed during the first quarter of the grant. Two of the siRNAs, siRNA-1 and siRNA2 at 15 nM, 30 nM and 60 nM concentrations were very effective in reducing levels of TGFb1 protein in the conditioned medium. Similarly, siRNA-1 and siRNA-2 at concentration of 15 nM were both effective in knocking down TGFb1 protein in extracts of the rabbit corneal fibroblast cell cultures. In contrast, siRNA3 was not as effective as the other two siRNAs

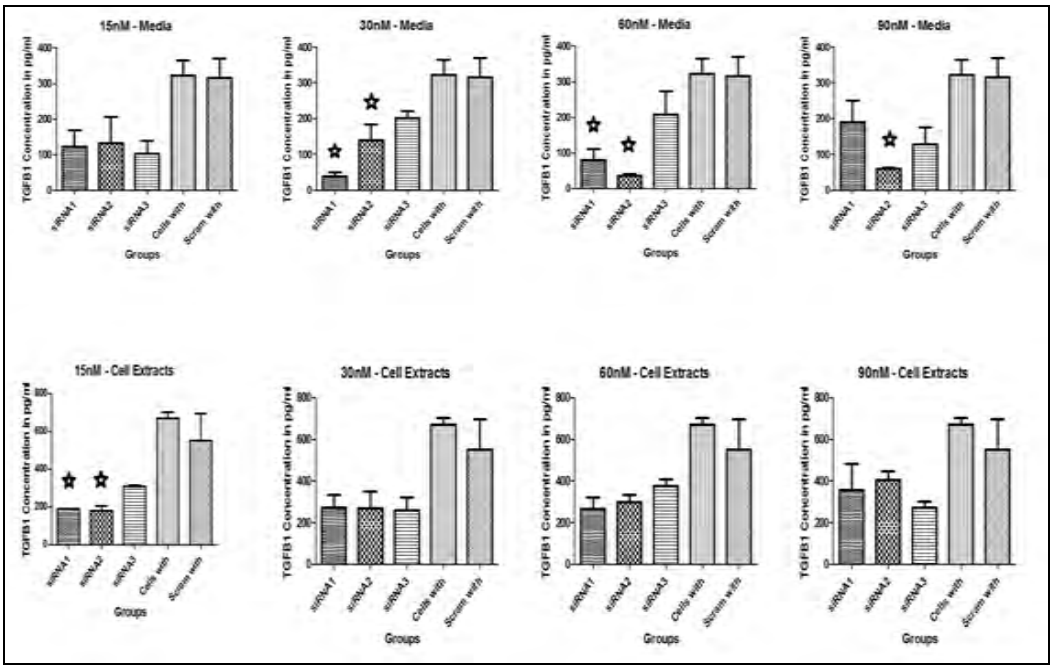

Figure 2. siRNA Knockdown of TGFb1 Protein. *Levels of TGFb1 protein were measured using ELISA in conditioned media and in extracts of low passage rabbit corneal fibroblasts transduced with increasing concentrations of three different siRNAs targeting TGFb1 mRNA. Both siRNA-1 and siRNA-2 significantly reduced levels of TGFb1 protein in conditioned media and cell extracts at low concentrations (15 to 30 nM).*
As shown in Figure 3, siRNA-1 and siRNA 2 met the go/no go cutoff of reducing TGFb1 protein by 80% (arrows) and so were selected to move to the next stage of drug development in testing double and triple combinations of siRNAs. The siRNA3 did not achieve 80% reduction so it was not further evaluated.

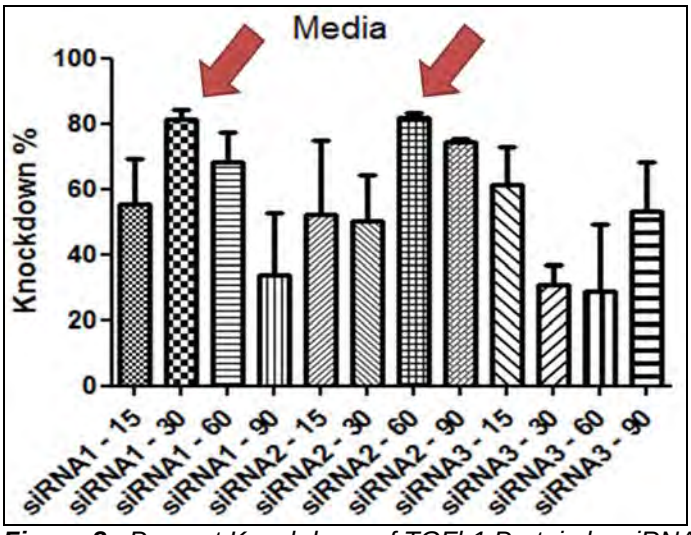

Figure 3*. Percent Knockdown of TGFb1 Protein by siRNAs in Culture Medium of Rabbit Corneal Fibroblasts*.

We also tested the siRNAs for knockdown of TGFb1 mRNA in cultures of rabbit corneal fibroblasts using Q-RT-PCR assays. As shown in Figure 4, levels of mRNA for TGFb1 was reduced by >90% in cultures of cells treated with all three of the siRNAs. In contrast, cells transfected with the scrambled siRNA control showed no knockdown of TGFb1mRNA.

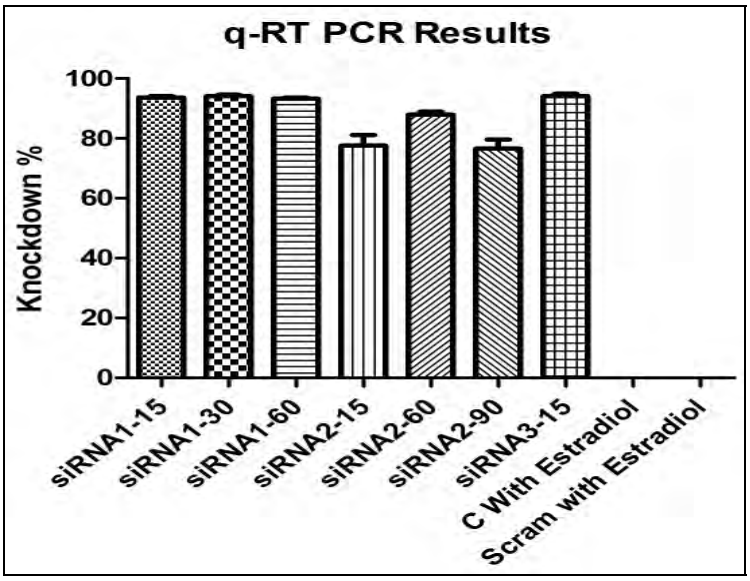

Figure 4. *Percent Knockdown of TGFb1 mRNA by siRNAs in Cultures of Rabbit Corneal Fibroblasts.*

<u>Objective 1f. Test up to four siRNA oligonucleotides (22mers) that selectively target mRNA for CTGF in low passage cultures of rabbit corneal fibroblasts for knockdown of CTGF protein and mRNA.</u>

Similar experiments were performed to test the knockdown of three siRNAs targeting CTGF mRNA and protein in cultures of rabbit corneal fibroblasts. As shown in Figure 5 below, only siRNA3 at concentration of 60 nM significantly reduced levels of CTGF protein in conditioned media of cultures of rabbit corneal fibroblasts that were stimulated by TGFb1 (which induces CTGF mRNA and protein).

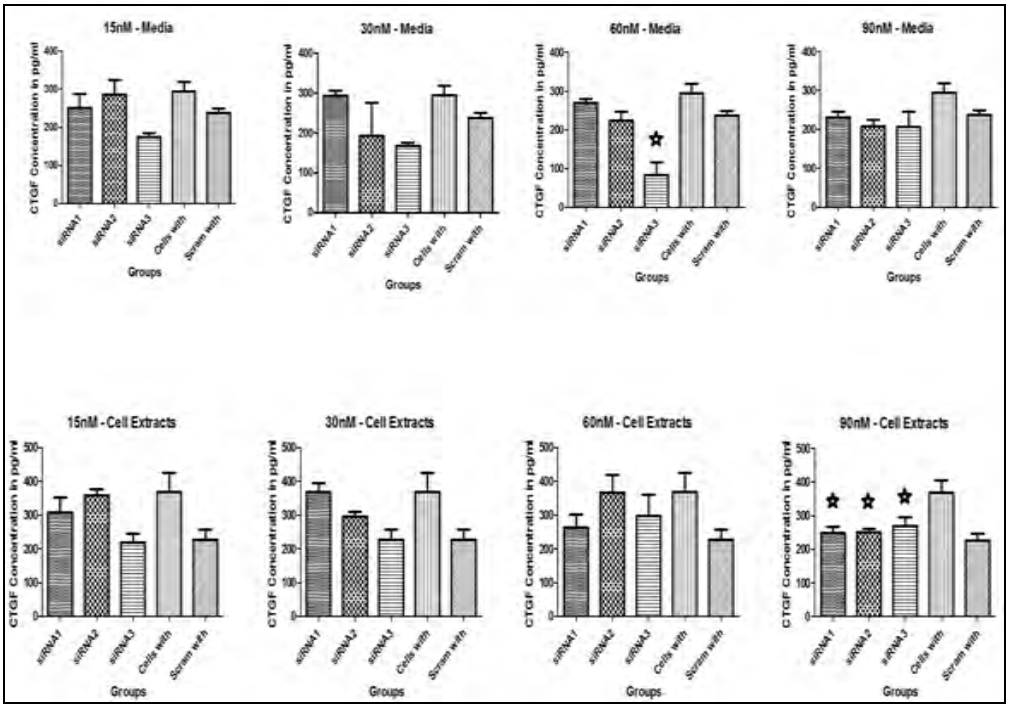

Figure 5. *Knockdown of CTGF Protein in Conditioned Media and Cell Extracts by siRNAs.*

As shown in Figure 6 below, siRNA3 targeting CTGF mRNA produced the best percent knockdown of CTGF protein. However, the level of knockdown was ~75%, which was slightly below the go/no threshold level that was set at 80%.

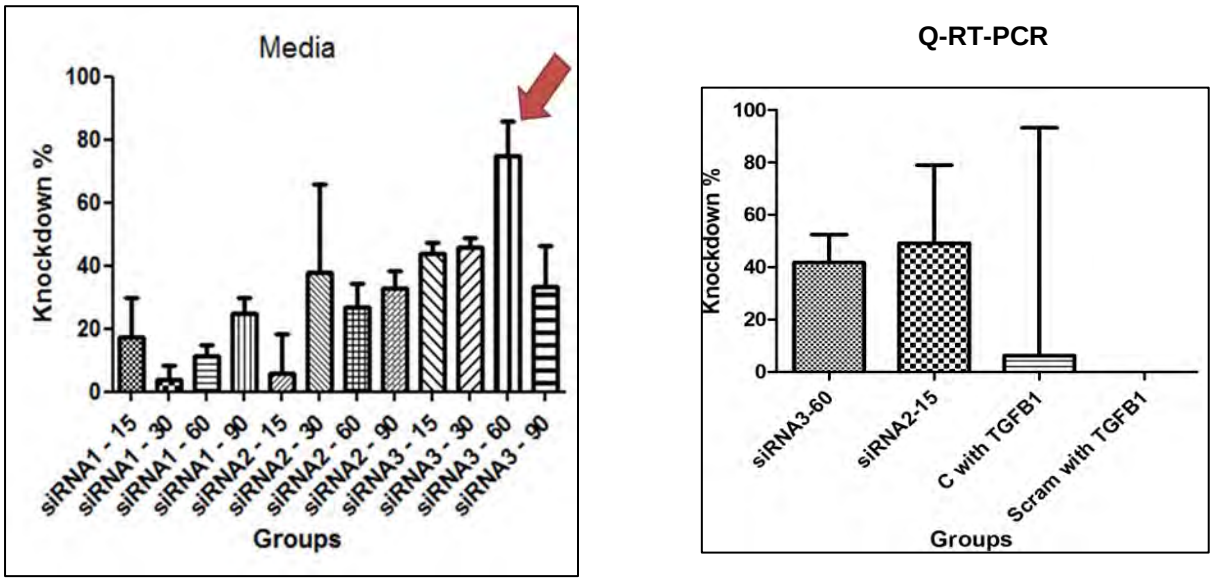

Figure 6. *Percent Knockdown of CTGF Protein and mRNA by siRNAs Targeting CTGF mRNA in Cultures of Rabbit Corneal Fibroblasts.*

<u>Objective 1g. Test two additional siRNA oligonucleotides (22mers) that selectively target mRNA for CTGF in low passage cultures of rabbit corneal fibroblasts for knockdown of TGFb1 protein and mRNA.</u>

In previous experiments, we identified one siRNA targeting CTGF mRNA that knocked down CTGF protein ~60% so we designed two new siRNAs targeting CTGF mRNA. As shown in Figure 7, two additional siRNAs targeting CTGF mRNA were tested for knockdown of CTGF protein using the optimized transfection conditions that were established in previous experiments. Both of the siRNAs (siRNA-1 and siRNA-2) produced significant knockdown of CTGF protein: siRNA-1 reduced CTGF protein the cell extract ~70% when added at 15 nM concentration and 60 nM; siRNA-2 added at 15 nM concentration reduced levels of CTGF protein ~80% in the conditioned medium of the estrogen stimulated rabbit corneal

fibroblasts; siRNA-2 added at 60 nM also showed good knockdown of CTGF protein (~65%) in both conditioned medium and cell extract. Based on these results, we focused on siRNA-2 targeting CTGF.

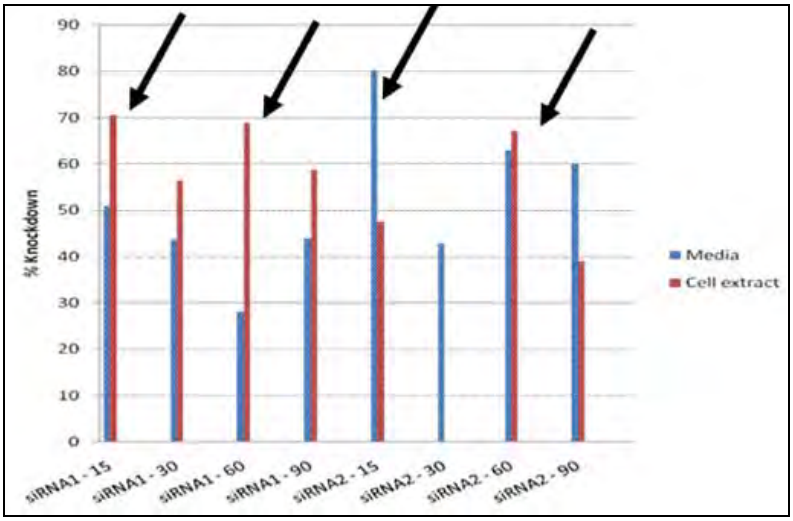

Figure 7. *Both siRNA-1 and siRNA-2 targeting CTGF mRNA significantly knocked down levels of CTGF protein in cultures of rabbit corneal fibroblasts stimulated by TGFb1.*

Objective 1h. Test siRNA oligonucleotides (22mers) that selectively target mRNA and protein for TGFbRII in low passage cultures of rabbit corneal fibroblasts.

Similar experiments were performed to test the knockdown of three siRNAs targeting TGFbRII mRNA and protein in cultures of rabbit corneal fibroblasts. As shown in Figure 8 below, both of the siRNAs achieved >80% knockdown of TGFbRII protein in conditioned media at concentrations of 15nM and 30nM in cultures of rabbit corneal fibroblasts that were stimulated by estrogen, which induces TGFbRII mRNA and protein.

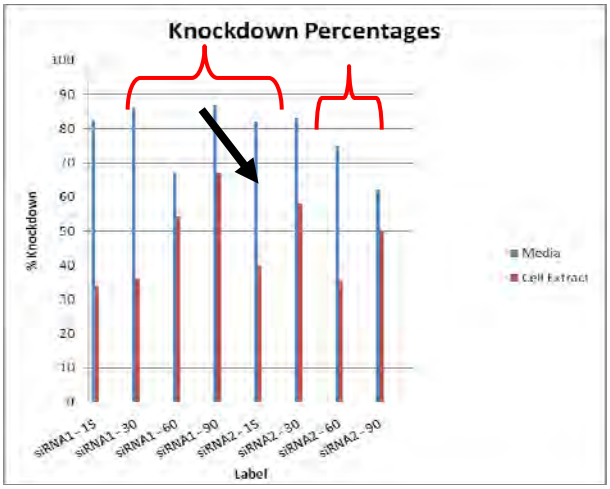

Figure 8. Individual TGFBR2 siRNA knockdown percentages.

Table 3. Nucleotide sequences of siRNAs targeting CTGF and TGFbRII

Growth Factor	siRNA sequence
TGFbR2	GCAGAGAACTTGAAAGCAT
TGFbR2	CCATATGCGGTGTGAAATA
CTGF	GUGAUGAGCCCAAGGACCA
CTGF	GCGAGGAGUGGGUGUGUGA

Objective 1g. Test the combination of two siRNA oligonucleotides targeting two separate target genes on the level of TGFb1 protein and mRNA in low passage cultures of rabbit corneal fibroblasts.

One of our key hypotheses is that the combination of two or more siRNAs targeting multiple genes in the TGFb-TGFbRII-CTGF system pathways would have the optimal knockdown of a target gene, like TGFb1. To test this hypothesis, we first needed to identify the siRNAs that had optimal knockdown of each of the separate genes, which we completed during the third quarter of the project.

The results of the first of several experiments combining siRNAs targeting different genes is shown in Figures 9 and 10. The two optimal siRNAs targeting TGFb1 (designated T1 and T2) and the two most effective siRNAs targeting TGFbRII (designated R1 and R2) were added alone (at 60 nM concentration) and in combinations (each at 30 nM for a final combined concentration of 60 nM) to cultures of rabbit corneal fibroblasts and the level of TGFb1 protein were measured using ELISA for TGFb1. Controls included adding each of the four siRNAs separately with the negative control scrambled ASO (designated NC). The combination of siRNAs T1 and R1 produced a knockdown of ~80% of TGFb1 protein in the conditioned medium of rabbit corneal fibroblasts. This was ~20% greater total knockdown than was generated by the effects of either siRNA added separately. Thus, these data supported our hypothesis that the combination of two siRNAs targeting two different genes, TGFb1 and TGFbRII, would produce significantly better knockdown than ether siRNA alone. The effect of the combination of T1+R1 siRNAs was not synergistic or additive, but the knockdown was significantly better than was achieved with either siRNA alone.

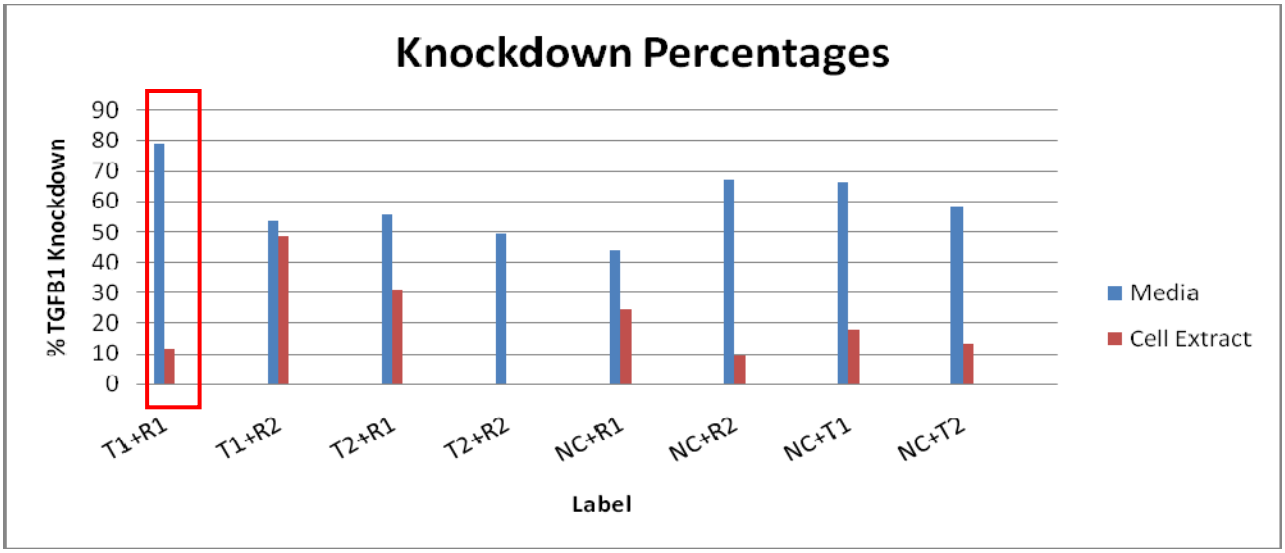

Figure 9. Knockdown percentages of TGFb1 protein for combined TGFbR2 + TGFb1siRNAs. The combination of T1+R1 siRNAs was more effective than either siRNA alone and more effective than other combinations of siRNAs.

Since the percent knockdown of TGFb1 protein does not provide information on the absolute levels of TGFb1 protein in the conditioned medium and in the cell extract, we also re-plotted the results for the combination of two siRNAs on TGFb1 using the actual amount of TGFb1 protein in the 96 well plate (the volume of conditioned medium and cell extract were both 150 ul so concentration is the same as total pg/well). As shown in Figure 4, the combination of T1+R1 siRNAs reduced the total amount of TGFb1 protein to levels that were essentially equal to unstimulated cells. In other words, near baseline levels of TGFb1 protein.

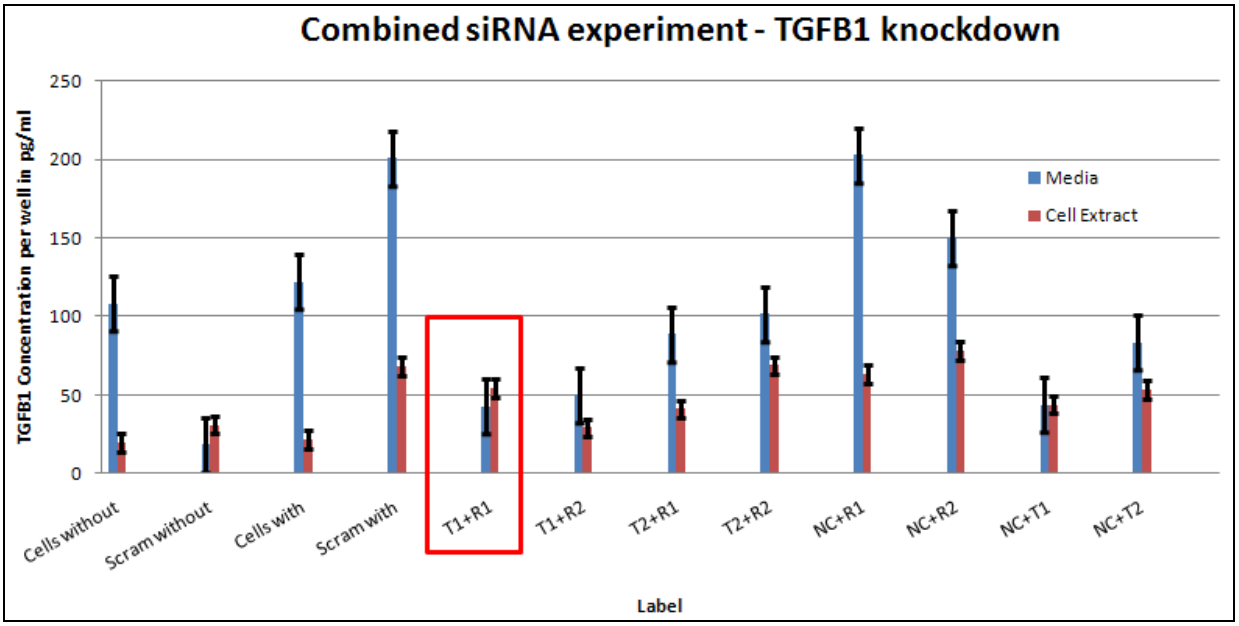

Figure 10. Concentration of TGFb1 protein in cells and medium of rabbit corneal fibroblasts treated with single or combined TGFbR2 + TGFb1siRNAs. The combination of T1+R1 siRNAs was more effective than either siRNA alone and more effective than other combinations of siRNAs.

The addition of siRNAs alone (at 60 nM) or in combinations (each siRNA at 30 nM) was tested for toxic effect on low passage cultures of rabbit corneal fibroblasts. As shown in Figure 11, cell viability was not reduced by addition of single siRNAs or combinations of siRNAs, at final concentrations of 60 nM siRNA oligonucleotides. Thus, the reduction in levels of target proteins and mRNAs was not due to general toxicity due to off-target effects of the siRNAs. This is an important control to perform to understand the knockdown effects and to perform before we begin tests in rabbit corneas following excimer laser ablation.

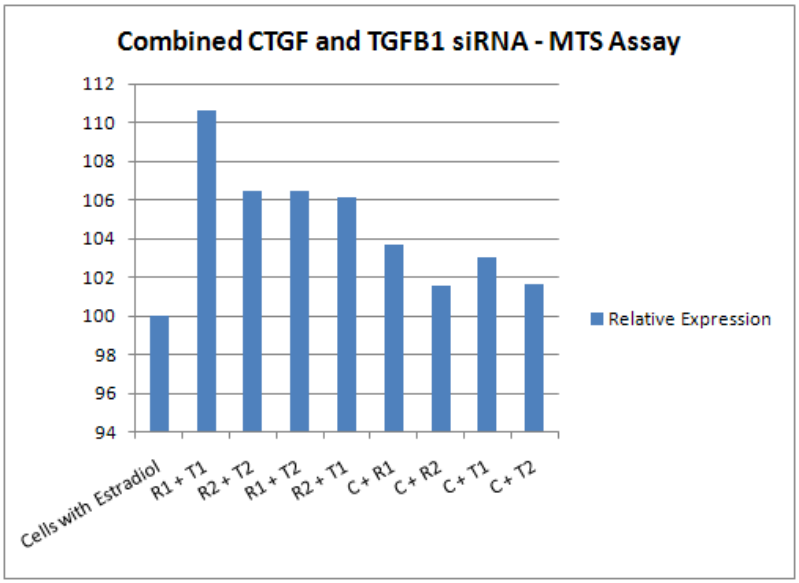

Figure 11. Assessment of cytotoxicity of siRNAs on rabbit corneal fibroblasts. Addition of single siRNAs at 60nM or combinations of siRNAs at 30 nM each did not reduce cell viability as measured by the MTS assay.

Similar experiments were performed using double combinations of siRNAs to TGFb and TGFbRII, and double combinations of TGFb and CTGF siRNAs as shown below in Figures 12, 13 and 14. In all these double combination experiments, optimal combinations of siRNAs were identified for knock down of TGFb, TGFbRII and CTGF mRNAs and proteins.

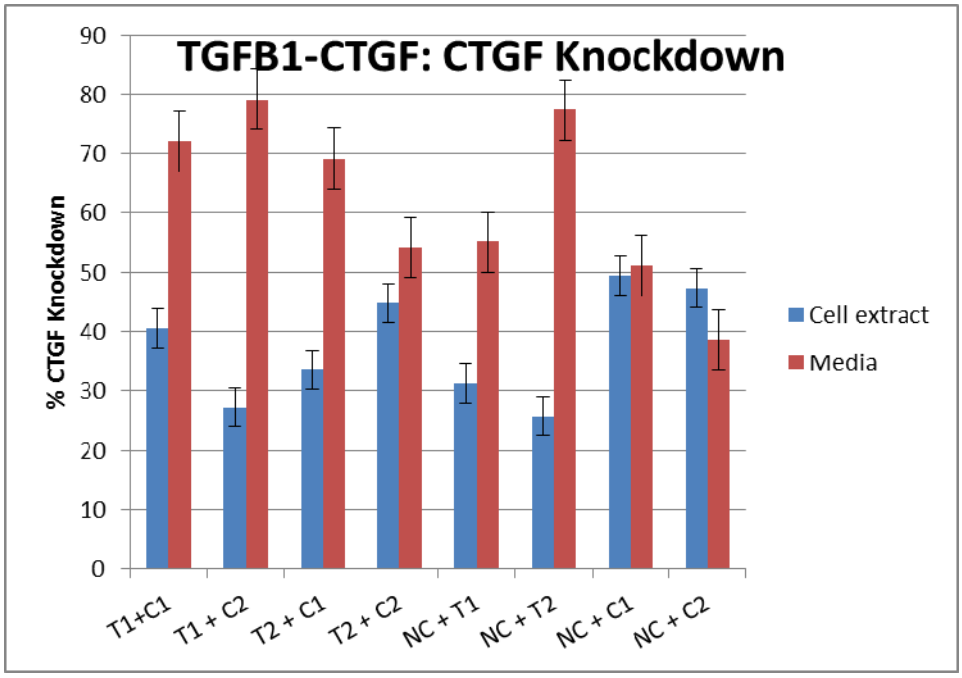

Figure 12. Protein level CTGF knockdown measured by ELISA. The CTGF knockdown % of TGFB1 and CTGF combination siRNA sequences (30nM total concentration) is compared with individual siRNA sequences (combined with scrambled to get 30nM total concentration).

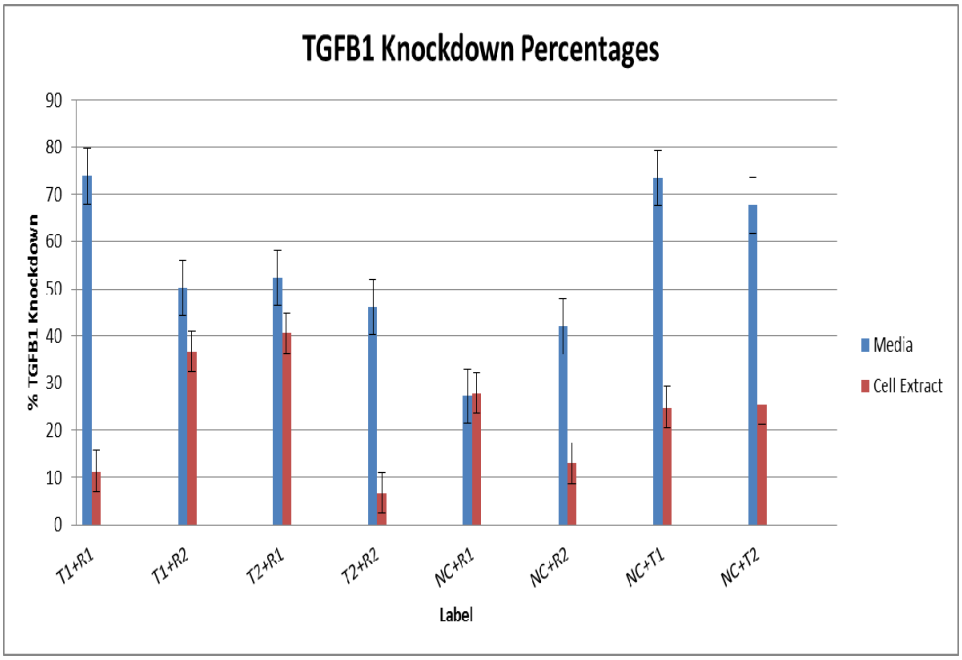

Fig 13. Protein level TGFB1 knockdown measured by ELISA. TheTGFB1 knockdown % of TGFB1 and TGFBR2 combination siRNA sequences (30nM total concentration) is compared with their respective individual siRNA sequences (combined with scrambled to get 30nM total concentration).

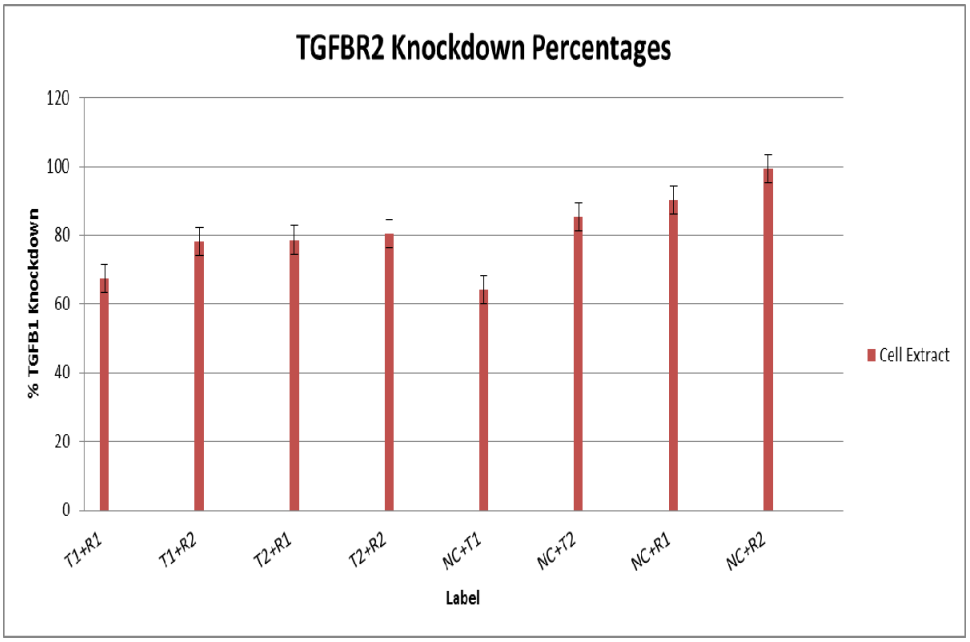

Fig 14. Protein level TGFBR2 knockdown measured by ELISA. TheTGFBR2 knockdown % of TGFB1 and TGFBR2 combination siRNA sequences (30nM total concentration) is compared with individual siRNA sequences (combined with scrambled to get 30nM total concentration).

Objective 1h. Identify the optimal triple combination of siRNAs targeting TGFb, TGFbRII and CTGF mRNAs based on effects of the combinations of two siRNA in low passage cultures of rabbit corneal fibroblasts for knockdown of collagen type I mRNA

The previous experiments identified the most effective single and double combinations of siRNAs targeting the three target genes. In the final series of *in vitro* experiments, we identified the best theoretical triple combination of siRNAs using the following mathematical equations.

- When two or more drugs are added in combination, one of the following three effects can be observed:
    - Additive (indifferent) effect: the activity of two drugs in combination is equal to the sum (or a partial sum) of their independent activity when studied separately
    - Synergistic effect: the activity of two drugs in combination is greater to the sum of their independent activity when studied separately
    - Antagonistic effect: the activity of two drugs in combination is less to the sum (or a partial sum) of their independent activity when studied separately

In order to construct the best triple combination, we have to analyze the results so that the presence of 1 siRNA sequence does not have an antagonistic effect on the other. The following are the basic equations involved in computing a Combination Index (CI) which would tell us if the sequences are synergistic, antagonistic or additive.

$$f_a = 1/[1 + (D_m/D)^m]$$

fa – is the fraction affected by siRNA seq (1/100 * knockdown %)
D – dose / concentration of the drug
Dm - median-effect dose (e.g., IC50, ED50, or LD50) that inhibits the system under study by 50%
m - coefficient signifying the shape of the dose-effect relationship

Combination Index:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = \frac{(D)_1}{(D_m)_1[f_a/(1-f_a)]^{1/m_1}} + \frac{(D)_2}{(D_m)_2[f_a/(1-f_a)]^{1/m_2}}$$

Where CI < 1 indicate synergism, CI = 1 indicates additive effect, and CI > 1 is antagonism. In the denominator, (Dx) is for D1 “alone” that inhibits a system x%, and (Dx)2 is for D2 “alone” that inhibits a system x%.

Table 4. Triple combination logic for maximum TGFB1 knockdown:

Drug	CI Values at					
	ED50	ED75	ED90	Dm	m	r
C1	N/A	N/A	N/A	1580.57311	-0.155	0.39475
(Not a combination)						
C2	N/A	N/A	N/A	66.64924	0.05718	0.08759
(Not a combination)						
R1	N/A	N/A	N/A	44.29044	0.79147	0.94924
(Not a combination)						
R2	N/A	N/A	N/A	8539.75283	0.03192	0.06065
(Not a combination)						
T1	N/A	N/A	N/A	86.27116	-0.50412	0.91994
(Not a combination)						
T2	N/A	N/A	N/A	12.15503	0.88476	0.80225
(Not a combination)						
T1C1	0.37864	31.28352	38786	30.97464	6.83312	1
(1:1)						
T1C2	0.75005	3.32399	33.79837	28.20254	7.84783	1
(1:1)						
T2C1	0.03828	0.01758	0.99459	0.46173	-0.36361	1
(1:1)						
T2C2	71.51209	512.33054	4339.85851	735.15832	0.32519	1
(1:1)						
T1R1	0.77998	3.74518	47.70293	22.82672	2.63302	1
(1:1)						
T1R2	0.2399	2.89268	35.23193	20.48946	3.4277	1
(1:1)						
T2R1	2.16929	0.83737	0.32431	20.68972	3.43782	1
(1:1)						
T2R2	1.66514	0.65262	0.25614	20.21109	3.58495	1
(1:1)						

Hence, combining T1-C1-R2 would give us the best effect.

Table 5. Triple combination logic for maximum CTGF knockdown:

Summary table						
Drug	Combination Index Values at					
	ED50	ED75	ED90	Dm	m	r
T1	N/A	N/A	N/A	86.27116	-0.50412	0.91994
(Not a combination)						
T2	N/A	N/A	N/A	12.15503	0.88476	0.80225
(Not a combination)						
C1	N/A	N/A	N/A	1580.573	-0.155	0.39475
(Not a combination)						
C2	N/A	N/A	N/A	66.64924	0.05718	0.08759
(Not a combination)						
R1	N/A	N/A	N/A	44.29044	0.79147	0.94924
(Not a combination)						
R2	N/A	N/A	N/A	8539.753	0.03192	0.06065
(Not a combination)						
C1T1	0.175	1.45446	181.3983	14.31614	-0.51436	1
(1:1)						
C1-T2	1.21267	3.61531	1327.71	14.62755	-0.95457	1
(1:1)						
C2T1	0.36642	0.59505	2.21716	13.77753	-1.2717	1
(1:1)						
C2-T2	0.71327	0.00012	2.42E-08	7.33253	-0.15104	1
(1:1)						
C1-R1	0.56457	3.72344	12058	24.32349	-3.6375	1
(1:1)						
C1-R2	0.01965	4.00818	11836	26.20443	-3.1518	1
(1:1)						
C2-R1	0.51779	0.03272	0.00344	13.77753	-1.2717	1
(1:1)						
C2-R2	0.49416	1.56E-09	4.98E-18	32.68037	-3.1363	1
(1:1)						

Hence, combining T1-C1-R2 would give us the best effect.

Table 6. Triple combination logic for maximum TGFBR2 knockdown:

Summary table						
Drug	Combination Index Values at					
	ED50	ED75	ED90	Dm	m	r
T1	N/A	N/A	N/A	86.27116	-0.50412	0.91994
(Not a combination)						
T2	N/A	N/A	N/A	12.15503	0.88476	0.80225
(Not a combination)						
C1	N/A	N/A	N/A	1580.573	-0.155	0.39475
(Not a combination)						
C2	N/A	N/A	N/A	0.48728	2.17234	0.99769
(Not a combination)						
R1	N/A	N/A	N/A	44.29044	0.79147	0.94924
(Not a combination)						
R2	N/A	N/A	N/A	8539.753	0.03192	0.06065
(Not a combination)						
R1-T1	1.42901	2.72225	13.75632	41.8212	-2.1659	1
(1:1)						
R2-T1	0.44168	3.17428	23.04343	37.72317	-5.5779	1
(1:1)						
R1-T2	8.48091	1.03333	0.12632	80.88714	-1.318	1
(1:1)						
R1-C1	31.95939	72.49621	5944.328	1376.912	-0.41089	1
(1:1)						
R2-C1	0.01775	1.08717	29684	23.67703	6.78655	1
(1:1)						
R1-C2	59.17178	24.24457	9.97099	28.51957	-2.8901	1
(1:1)						
R2-C2	1.52796	0.31762	0.06603	0.74451	-1.0315	1
(1:1)						
R2-T2	3.21598	0.75635	0.17814	39.03471	-5.3786	1
(1:1)						

Hence, combining T1-C1-R2 would give us the best effect.

Table 7. Comparison of the collagen knockdown % of the siRNA triple combination made from the sequences T1, C1 and R2 to their respective individual sequences.

T1C1R2 effect on Collagen knockdown

	T1 alone %	C1 alone %	R2 alone %				T1C1R2 %
10nM	48.8	98.9	86			15nM	18.1
15nM	0	0	94.32			30nM	83.1
20nM	27.6	85.32	81.8			60nM	97.1
30nM	82.8	70.46	42			90nM	88.04

As shown in Figure 15, the percent knock-down of TGFbRII and CTGF mRNAs by the triple combination of siRNAs was very good at approximately >95% when the three siRNAs were added at 30 nM eack.

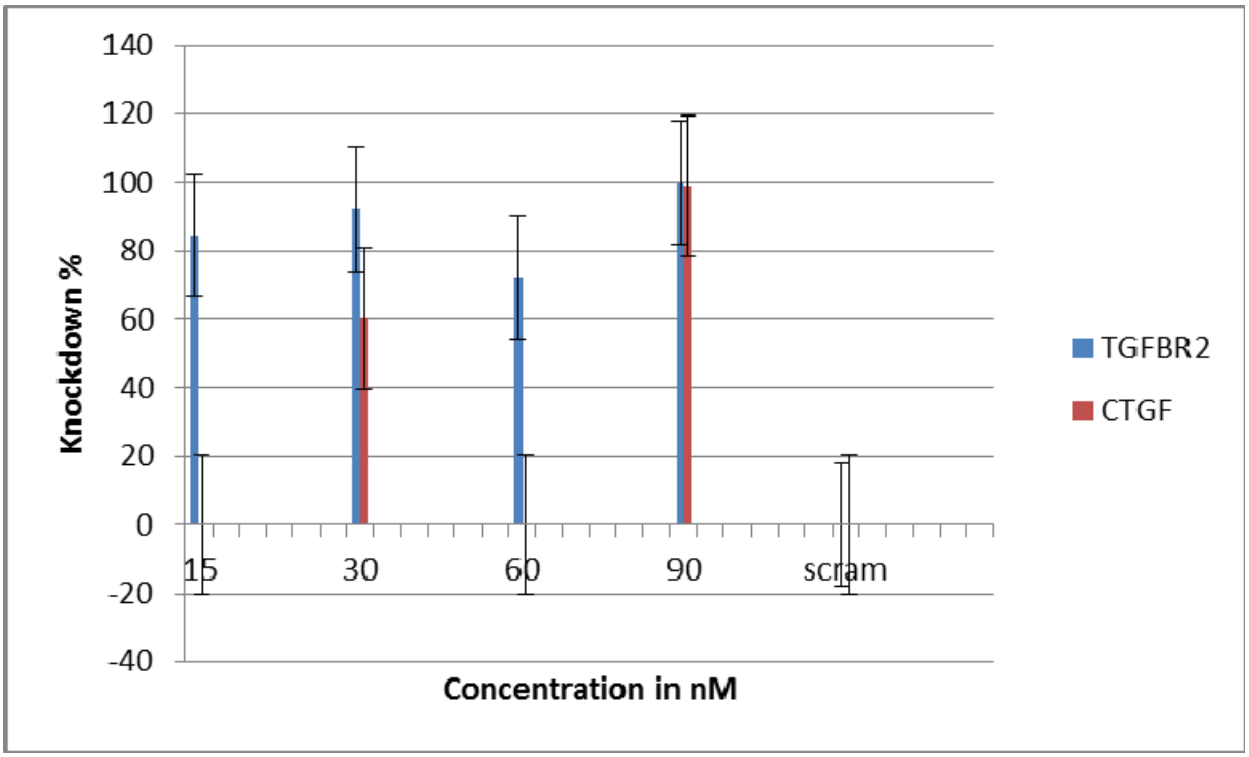

Figure 15. RNA levels of TGFbRII and CTGF knockdown measured by q RT PCR. TGFbRII and CTGF knockdown percent of the triple combination made from siRNA sequencesT1,C1 and R2 are calculated at different total siRNA concentrations.

In Figure 16 shown below, the level of type I collage mRNA knock down by each of the separate siRNAs at 30 nM was 40% for R2 siRNA; was 70% for C1 siRNA; and was 80% for T1 siRNA. Thus, none of the individual siRNAs achieved over 80% knock down alone of the type I collagen mRNA. In marked contrast, as shown in Figure 17, the triple combination of the three siRNAs (T1, R2 and C1) each at 20 nM cause a remarkable 97% knock-down of the mRNA for type I collagen mRNA.

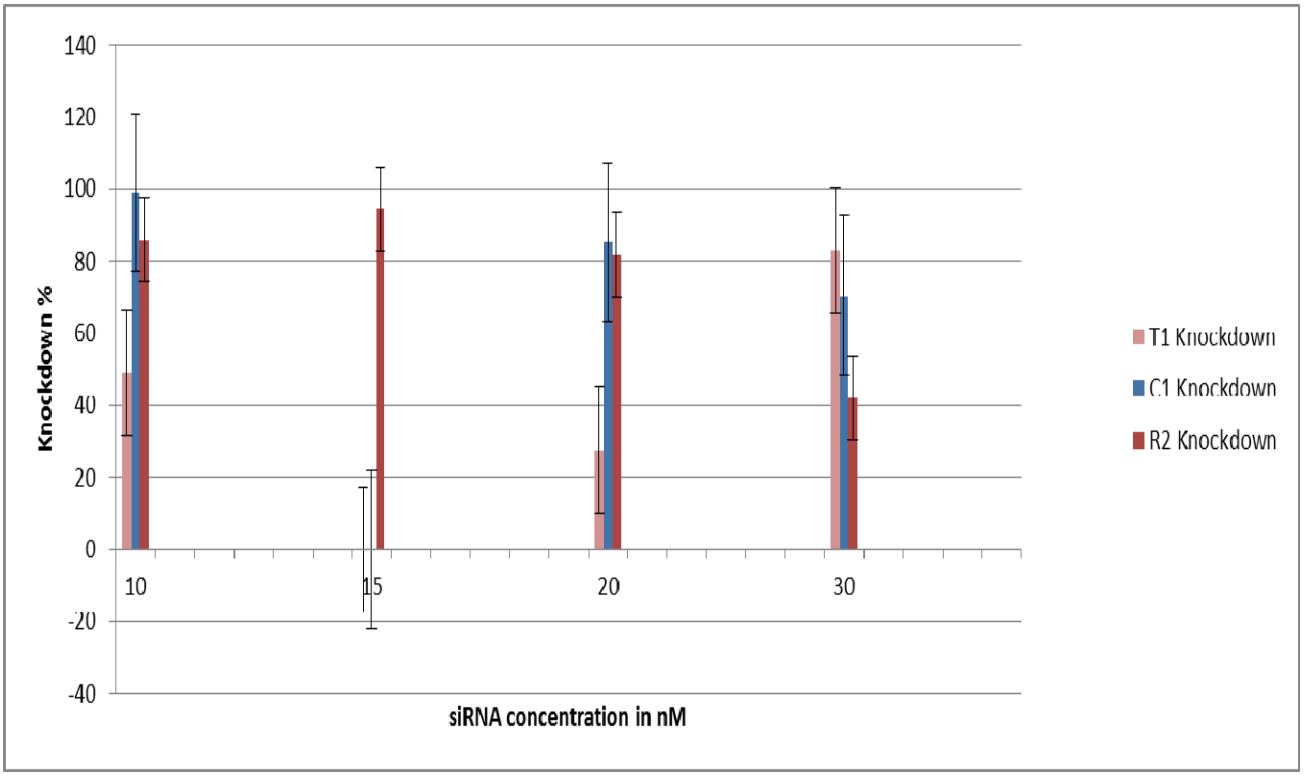

Figure16. RNA level Collagen knockdown measured by q RT PCR. Collagen knockdown percent of the individual siRNA sequencesT1,C1 and R2 are calculated at different total siRNA concentrations.

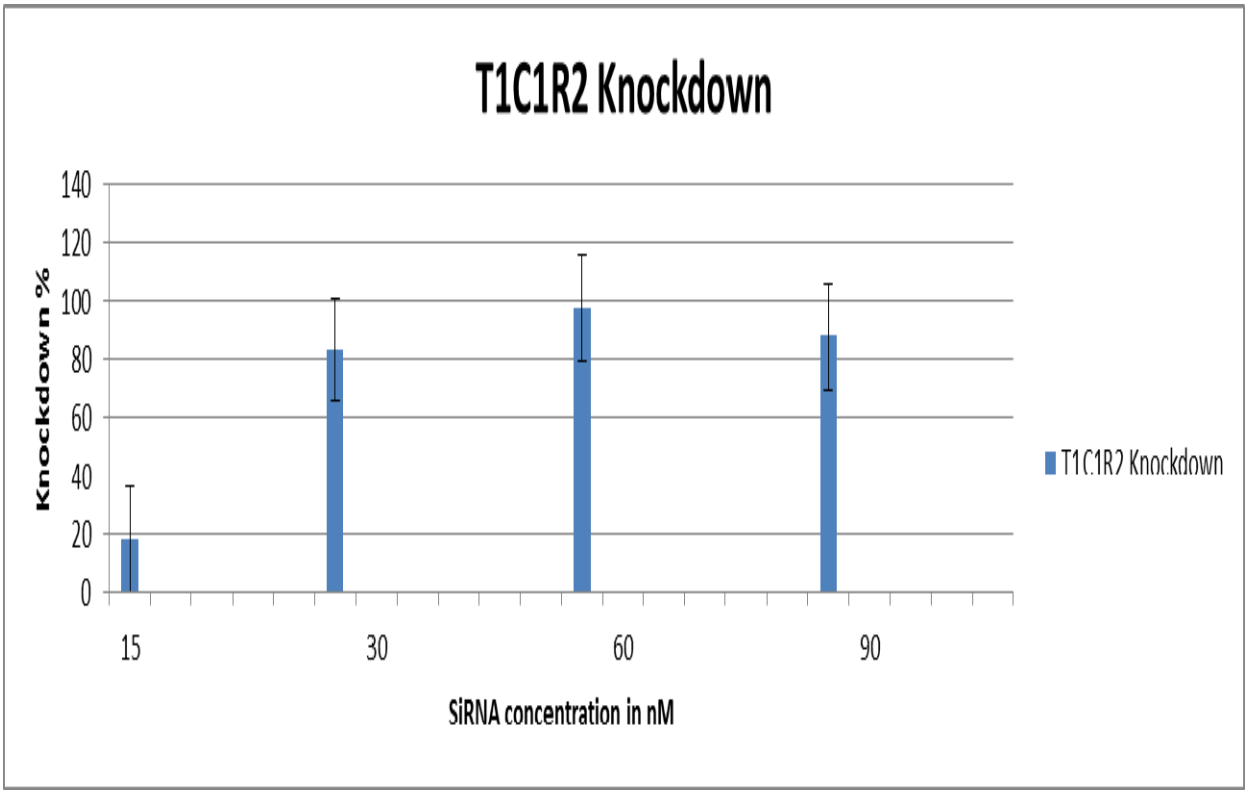

Figure 17. RNA level Collagen knockdown measured by q RT PCR. Collagen knockdown percent of the triple combination made from siRNA sequencesT1,C1 and R2 are calculated at different total siRNA concentrations.

In summary, we successfully identified a triple combination of siRNAs targeting TGFb, TGFbRII, and CTGF mRNAs and achieved a remarkable 97% knockdown of collagen type I mRNA in cultures of corneal fibroblasts when the siRNAs were added at 20 nM concentrations.

Objective 1i. Optimize the animal model of blast injury to cornea and develop a method to measure amount of corneal haze (scar) that occurs in the rabbit cornea.

As shown in Figure 18 below, corneas injured with a large (6 mm diameter) central ablation created with an excimer laser begin to develop visible haze (scar) beginning about day 6 after injury and the extent of corneal haze continues to increase over the next several days. Control corneas that were not injured with the excimer laser remained clear.

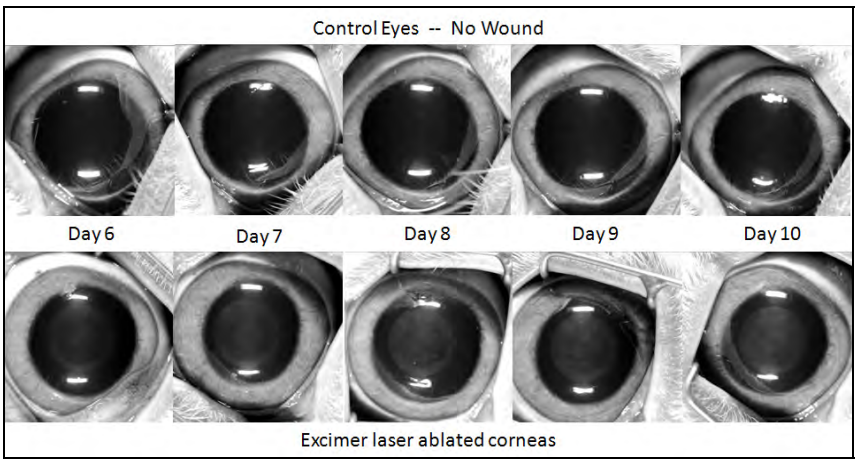

Figure 18. Photographic images of rabbit corneas injured with a large central ablation wound developed progressively more severe corneal haze (scar) while control rabbit corneas remained clear.

As shown in Figure 19, using this standardized photographic technique the digital images were processed by software (Photoshop) and converted into false color maps with integration of total light scatter pixel intensity in the area of the ablation. This method now allows us to quantitatively measure the level of light scatter which is clinically described as haze, and it will be used to assess the effects of the siRNAs on reducing corneal scarring (haze) on rabbit corneas following blast and burn injuries.

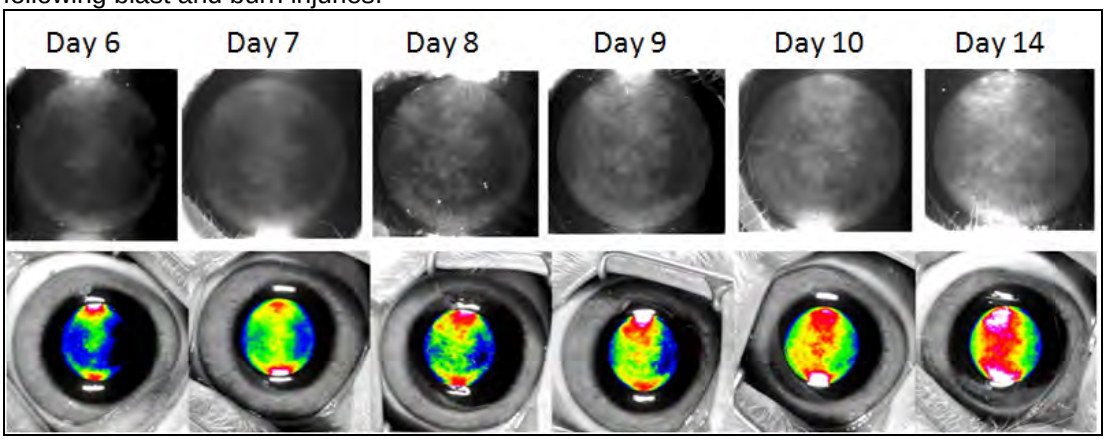

Figure 19. Quantitative analysis of photographic images of rabbit corneas following excimer laser ablation will enable us to assess the effects of the siRNA treatments on reducing corneal haze (scar) following blast and burn injuries to rabbit corneas.

KEY RESEACH ACCOMPLISHMENTS

- Optimal siRNAs were identified for each of the three key target genes, TGFb, TGFbRII, and CTGF that produced at least 70% knock down of target mRNAs and similar knock down of the target proteins

- Optimal double combinations of siRNAs were identified that produced greater than 80% knock down of at least one of the three target gene mRNAs

- An optimal triple combination of siRNAs was identified that produced 97% knock down of mRNA for type I collagen mRNA in RCF cultures

- The excimer laser ablation model for rabbit corneas was optimized along with a quantitative image analysis system to measure levels of corneal scarring as indicated by light scattering, which is clinically called corneal haze

REPORTABLE OUTCOMES

The results of these experiments will be presented as an abstract at the 2012 annual meeting of the Association for Research in Vision and Ophthalmology and at the 2012 annual meeting of the Wound Healing Society. In addition a manuscript is being written for submission to an appropriate research journal like Nature Biotechnology.

CONCLUSIONS

The results of the experiments conducted during the first year of this two year research project have achieved an important milestone, which is the identification of a triple combination of siRNAs targeting three key genes that reduces the level of mRNA for type I collagen gene by 97%. This triple combination achieves this dramatic effect by a nealy synergistic reduction of the levels of mRNAs for each of these three key gene that promote corneal scarring, specifically TGFb, TGFbRII, and CTGF.

The results of these experiments hold great promise for a new drug formulation that will reduce corneal scarring without significant detrimental side effects that are produced by other drugs like mitomycin-C or 5-fluoruracil that are used in desperation to reduce corneal scarring

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APPENDICES

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